Apoptosis

CardioTACS™ *In Situ* Apoptosis Detection Kit

Catalog Number: 4827-30-K

Reagent kit for *in situ* detection of apoptosis in cardiac tissue sections and cells.

30 samples

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Apoptosis is characterized by a number of intracellular phenomena such as membrane blebbing, chromatin condensation and nuclear DNA fragmentation. Detection of nuclear DNA fragmentation is a widely accepted method to assay for apoptosis and can be performed *in situ* by incorporating labeled nucleotides onto the free: 3' OH ends of DNA fragments using a terminal deoxynucleotidyl transferase (TdT) Enzyme. Terminal dUTP nick-end labeling (TUNEL) allows for the monitoring of apoptotic events in cells and within tissue sections. The incorporation of biotinylated nucleotides allows chromosomal DNA fragmentation to be visualized by binding streptavidin-horseradish peroxidase followed by reaction with TACS Blue Label™ to generate a dark blue precipitate. When viewed under a standard light microscope, apoptotic cells are clearly distinguished by the dark blue staining.

PRINCIPLE OF THE ASSAY

CardioTACS *In Situ* Apoptosis Detection Kit provides all the reagents for the detection of DNA fragmentation in cardiac cells and tissue sections. This kit is ideal for the detection of apoptosis in frozen samples, paraffin-embedded cardiac specimens, and cells in culture. The CardioTACS *In Situ* Apoptosis Detection Kit is supplemented with a cation that specifically enhances the signal in apoptotic cardiac cells. In addition, the TACS-Nuclease™ provided in this kit allows for positive controls to be generated for each experiment.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each sample level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTIONS

The acute and chronic effects of overexposure to reagents of this kit are unknown. Safe laboratory procedures should be followed, and protective clothing should be worn when handling kit reagents.

The CardioTACS™ *In Situ* Apoptosis Detection Kit contains reagents that are harmful if swallowed or in contact with skin, and irritating to the eyes, respiratory system, and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
Cytonin™	4876-05-01	6.0 mL	Store 2-8 °C.
10X TdT Labeling Buffer	4810-30-02	100 mL	
10X TdT Stop Buffer	4810-30-03	100 mL	
Strep-HRP	4800-30-06	30 µL	
TACS Blue Label	4800-30-11	1.5 mL	
Blue Streptavidin-Diluent	4800-30-12	7.5 mL	
TACS-Nuclease Buffer	4800-30-16	1.5 mL	
Proteinase K	4800-30-01	50 µL	Store at ≤ -20 °C.
TdT dNTP Mix	4810-30-04	35 µL	
TdT Enzyme	4810-30-05	30 µL	
50X Mn^{2+}	4810-30-14	30 µL	
TACS-Nuclease	4800-30-15	15 µL	
Nuclear Fast Red	4800-30-17	50 mL	Room Temperature

OTHER MATERIALS REQUIRED

Equipment:

- Pipette and pipette tips
- 37 °C incubator
- 50 mL and 500 mL graduated cylinders
- 2 Coplin jars
- -20 °C and 2-8 °C storage
- Ice bucket
- Standard light microscope
- Cryostat or microtome
- Humidity chamber
- 57 °C incubator or slide warmer
- Timer

Reagents:

- Distilled water
- 10X Phosphate Buffered Saline (PBS)
- 37% formaldehyde
- o- or p- xylenes
- 30% hydrogen peroxide
- 70%, 95% and 100% ethanol
- Methanol
- Permount™ Mounting Medium

Disposables:

- Treated glass microscope slides (or alternative support)
- 50 mL tubes
- Microcentrifuge tubes
- 1.5 mL and 10 mL serological pipettes
- Gloves
- Hydrophobic coverslips (optional)
- Glass coverslips

REAGENT PREPARATION

The volumes given for each reagent are based on processing up to 4 cm^2 samples that are immobilized on glass slides. Different configurations of chamber slides, culture plates, free floating sections, or the use of glass coverslips may require adjustments to the stated volumes.

Reagents marked with an asterisk (*) should be prepared immediately before use.

1X PBS- Dilute 10X PBS 1:10 using distilled water. Store 1X PBS at room temperature. Approximately 500 mL of 1X PBS is used to process 1-10 slides.

***3.7% Buffered Formaldehyde -** 50 mL of freshly prepared fixative is used to process 1-10 samples. To prepare add:

Reaction Component	Volume
37% Formaldehyde	5.0 mL
10X PBS	5.0 mL
Distilled water	40 mL

Note: Wear gloves and exercise caution when handling formaldehyde solutions.

***Proteinase K Solution -** Use 50 µL of Proteinase K Solution per sample. Store on ice. Thaw provided Proteinase K at room temperature, then place on ice. To prepare add:

Reaction Component	2 Samples	10 Samples	n Samples
Distilled water	100 µL	500 µL	n x 50 µL
Proteinase K	2.0 µL	10 µL	n x 1.0 µL

Note: Under some circumstances, the Proteinase K may also be used at a 1:200 dilution

Cytonin - Cytonin is ready for use. If required, 50 µL of Cytonin is used per sample. Store at 2-8 °C. Discard if solution is cloudy.

***Quenching Solution -** Use 50 mL of Quenching Solution to process 1-10 samples. To prepare add:

Reaction Component	Volume
Methanol	45 mL
30% hydrogen peroxide	5.0 mL

Always use fresh 30% hydrogen peroxide. It is recommended that 6.0 mL aliquots of fresh 30% hydrogen peroxide be made and stored at 2-8 °C. For each labeling procedure, use a fresh 30% hydrogen peroxide aliquot then discard the unused portion.

1X TdT Labeling Buffer - Dilute the 10X TdT Labeling Buffer 1:10 using distilled water. Leave at room temperature until use. Use 50 mL of 1X Labeling Buffer to process 1-10 samples. Remove an aliquot of 50 µL per sample for preparing the Labeling Reaction Mix and place on ice.

REAGENT PREPARATION *Continued*

***Labeling Reaction Mix -** Thaw 50X Mn^{2+} and TdT dNTP Mix at room temperature, then place on ice. To maintain optimal enzyme activity, remove the TdT Enzyme tube from freezer only long enough to pipette the required volume. Alternatively, place the TdT Enzyme in a -20 °C freezer block. Prepare the Labeling Reaction Mix just before use and keep the prepared reaction mix on ice. Prepare one reaction mix without the enzyme.

Prepare 50 µL per sample in the sequence given below:

Reaction Component	2 Samples	10 Samples	n Samples
TdT dNTP Mix	2.0 µL	10 µL	n x 1.0 µL
TdT Enzyme	2.0 µL	10 µL	n x 1.0 µL
50X Mn^{2+}	2.0 µL	10 µL	n x 1.0 µL
1X TdT Labeling Buffer	100 µL	500 µL	n x 50 µL

1X TdT Stop Buffer - Dilute the 10X TdT Stop Buffer 1:10 using distilled water. Leave at room temperature until use. Use 50 mL of 1X TdT Stop Buffer to process 1-10 samples.

***Strep-HRP Solution** - Use 50 µL of Strep-HRP Solution per sample. Store prepared Strep-HRP Solution at room temperature until use. To prepare add:

Reaction Component	1-16 Samples
Blue Streptavidin-Diluent	800 µL
Strep-HRP	1.0 µL

***TACS Blue Label** - TACS Blue Label is ready to use. 50 µL of TACS Blue Label is used per sample. A faint blue coloration may be observed on storage which does not affect stability or activity. Store at 2-8 °C.

***TACS-Nuclease Solution**- For the preparation of a TACS-Nuclease-treated Positive Control Sample, it is recommended that the DNA breaks be generated in a separate step. In this case, TACS-Nuclease should be diluted 1:50 in TACS-Nuclease Buffer, as below:

Reaction Component	2 Samples	10 Samples	n Samples
TACS-Nuclease Buffer	100 µL	500 µL	n x 50 µL
TACS-Nuclease	2.0 µL	10 µL	n x 1.0 µL

It is also possible to obtain acceptable, but lower intensity, positive control staining by incubating the TACS-Nuclease with the Labeling Reaction Mix. In this case, prepare as below:

Reaction Component	Per Positive control
1X TdT Labeling Buffer	50 µL
TdT dNTP Mix	1.0 µL
TdT Enzyme	1.0 µL
TACS-Nuclease	1.0 µL
50X Mn^{2+} Cation	1.0 µL

REAGENT PREPARATION *Continued*

Nuclear Fast Red - The Nuclear Fast Red counterstaining solution is ready for use, and can be reused many times. Store in a closed container to prevent evaporation. If a precipitate is visible, solubilize at 37 °C before use.

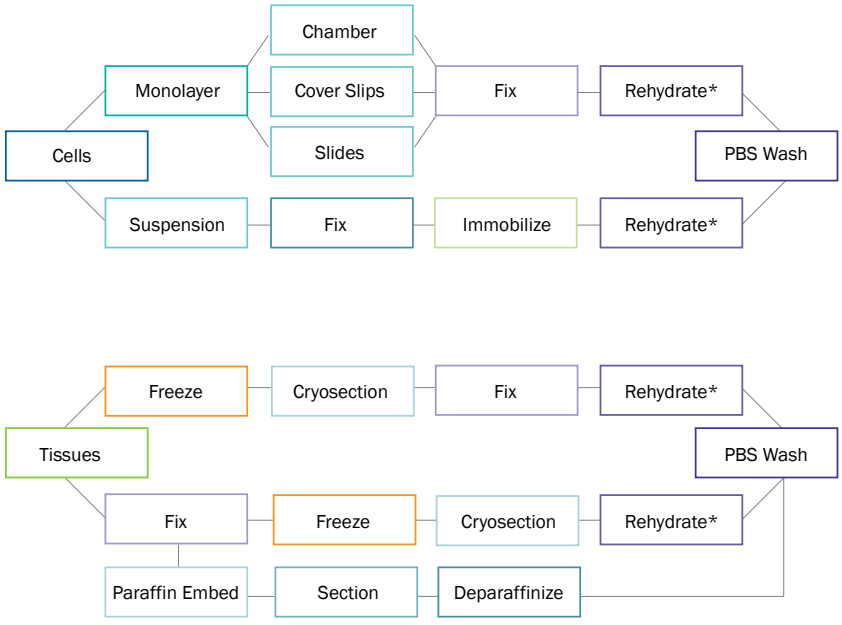
Xylenes - Mixed xylenes may be used for deparaffinization and for clarification prior to mounting coverslips onto the samples. Xylenes used for deparaffinization may be reused several times. Xylenes used in deparaffinization should not be used for clarification. Use ACS grade o- or p-xylene for clarification prior to mounting.

100%, 95%, 70% Ethanol - Either 100% (200 Proof) or denatured alcohol (90% ethanol, 5% methanol, 5% isopropanol) may be used. Dilute with distilled water to prepare 95% and 70% solutions. Ethanols used for deparaffinization may be reused several times. Ethanols used in deparaffinization should not be used for dehydration.

ASSAY PROTOCOL

It is important to read through the Assay Protocol before preparing tissue or cell samples for labeling. This section includes instructions for sample preparation, *in situ* labeling and viewing. The assay protocol for labeling is in tabulated form and details the steps involved in the labeling reaction and in preparing the sample for viewing. Prior to labeling, the samples must be rehydrated, if necessary, and washed in PBS. The labeling procedure begins with samples in PBS regardless of the fixation and immobilization method.

Follow the appropriate flow diagram to determine the correct sequence of steps needed to prepare samples. The tissue or cell type, source, storage conditions, facilities, and equipment available will help determine which method is most appropriate. In addition, careful consideration of each method's advantages and disadvantages should be made.



*Rehydration may not be required if samples are not dried.

SAMPLE PREPARATION AND FIXATION

PREPARATION OF CELLS IN MONOLAYER

On Sterile Chamber Slides - For optimal outcomes, cells should be grown on a surface that allows for fixation and direct labeling, such as sterile chamber slides. Remove the chamber walls and gasket after fixation. The chamber walls and gasket may be left in place during the labeling reaction if different treatments (i.e., no enzyme and nuclease treatment) are required for adjacent samples on the same slide.

On Sterile Slides - Other cell culture methods include culturing directly on microscope slides. The slides must be sterile and, if necessary, pretreated to ensure cell adhesion. Sterilize microscope slides by autoclaving in a large glass petri dish. If needed, coat slides with sterile poly-L-lysine or collagen, however, these slide pretreatments may increase background staining. Place sterile microscope slides in culture vessel directly before plating cells.

On Sterile Glass Coverslips - Cells can be cultured directly on sterile coverslips that are placed into a 12- or 24-well tissue culture plate. Sterilize coverslips by autoclaving in a large glass petri dish. If needed, coat coverslips with sterile poly-L-lysine or collagen, however, these slide pretreatments may increase background staining. Place sterile glass coverslips in wells of tissue culture dishes (12 mm coverslips fit into 24-well tissue culture plates) using fine tipped sterile forceps. Handle only at edges prior to cell plating.

1. Remove media from cells and rinse once with 1X PBS at room temperature.
2. Fix cells for 10 minutes at room temperature in 3.7% Buffered Formaldehyde.
3. Wash cells one time in 1X PBS. Samples can be stored at this point, using one of the following methods:

   a) Dehydrate the cells by passing through an increasing alcohol series of 70%, 95%, and 100% ethanol for 5 minutes each followed by air drying for 10 minutes. Store at 2-8 °C with desiccant. Rehydrate prior to labeling.

   b) Fixed cells can be stored for up to 1 week in Cytonin at 2-8 °C. The samples must be covered to prevent contamination and evaporation. If experimental design dictates a time course extending over several days, storage in Cytonin is recommended.

   Note: *Labeling directly after fixation is optimal as the labeling of some samples is less efficient after storage. If possible, a pilot study should be performed to ensure that stored fixed samples can be labeled.*

4. Proceed to Labeling Procedure.

SAMPLE PREPARATION AND FIXATION *Continued*

Storage

Note: *Labeling directly after fixation is optimal. The labeling of some samples is less efficient after storage. Empirical determination is required to determine if samples label efficiently after storage.*

Two storage methods for fixed samples are provided:

1. Fixed cells can be dehydrated and stored at 2-8 °C with desiccant for several months. After step 3, dehydrate by immersing for 5 minutes each in 70%, 95%, and then 100% ethanol, followed by air drying for 2 hours on a slide warmer at 45 °C. Samples that must be prepared weeks or months apart should be stored desiccated. If possible, a pilot study should be performed to ensure that stored fixed samples can be labeled.
2. Fixed cells can be stored for up to 1 week in Cytonin at 2-8 °C. The samples must be covered to prevent contamination and evaporation. If experimental design dictates a time course extending over several days, storage in Cytonin is recommended.

SAMPLE PREPARATION AND FIXATION *Continued*

PREPARATION OF TISSUES

Use of glass slides pretreated for electrostatic adherence is recommended for all tissues.

Preparation of Fresh Frozen Sections - Fresh tissue requires minimal fixation and frozen samples are easily permeabilized for labeling. Some disadvantages include the difficulty in collecting good quality sections, the need to cut thicker sections, and poor retention of morphology. Frozen sections are less resistant to protease treatments and can lift off if not prepared onto the appropriately pretreated slides and dried thoroughly. **Samples must be fixed prior to labeling.**

1. **Freeze Tissue** - Rapidly freeze tissue or biopsy immediately after removal by immersing in liquid nitrogen or on dry ice. Store frozen tissue ≤ -70 °C.

2. **Cryosection Frozen Tissue** -

   a. Samples may be embedded in a cutting matrix. Position the sample within cutting matrix in a suitable container. Immerse embedded tissue in isopentane chilled on dry ice. Frozen samples may be stored for many months at ≤ -70 °C.

   b. Using the cutting matrix, attach the sample to cutting block and equilibrate to the temperature of the cryostat before sectioning. Collect sections between 6-15 µm on glass slides pretreated for electrostatic adherence.

   c. Individual expertise and tissue type will determine the thickness of the sections. Sections between 10-15 µm provide the best results. Sections between 6-9 µm tend to tear during cutting, resulting in rough edges that can increase the background staining. Up to 3 sections can be placed per slide; each spaced well apart to prevent reagents from mixing between samples.

3. **Fixation After Cryosectioning**

   a. It is critical to dry the samples thoroughly after sectioning. Dry overnight at room temperature or for at least 2 hours at 45 °C on a slide warmer. Samples can be stored at this point. Store slides at ≤ -70 °C with desiccant for up to 3 months. After storage equilibrate samples to room temperature and re-dry for 2 hours at room temperature or 2 hours at 45 °C on a slide warmer.

   b. Rehydrate by immersing for 5 minutes each in 100%, 95%, then 70% ethanol.

   c. Wash once in 1X PBS for 5 minutes.

   d. Fix samples by immersing in 3.7% Buffered Formaldehyde for 10 minutes at room temperature.

   e. Wash cells 2 times in 1X PBS, 5 minutes each.

   f. Proceed to Labeling Procedure.

SAMPLE PREPARATION AND FIXATION *Continued*

Preparation of Fixed Samples Before Sectioning – Immersion or Perfusion

Samples are routinely fixed by immersion or perfusion methods. After fixation, samples are cryosectioned or paraffin embedded. Formaldehyde is the recommended fixative based on laboratory testing. Other fixatives that maintain DNA integrity may be used. These include other cross linking agents such as paraformaldehyde and glutaraldehyde. Bouin's reagent should be avoided due to the high acidity. If alternative fixatives are used, it is recommended that a pilot study is performed to ensure that the fixative allows for permeabilization and labeling. Regardless of the fixative used, it is important not to fix cells and tissues for extended periods of time.

Immersion Fixation - The fixation time should ensure good cross-linking but prevent tissue from becoming hard and brittle. Some empirical determination of the optimal fixation time may be required. Immerse relatively small pieces of tissue (1 cm^3) in at least 10 volumes of 3.7% Buffered Formaldehyde. After 30 minutes change to fresh fixative and leave at room temperature up to 24 hours. Tissues with high cellularity may require longer fixation times.

Perfusion Fixation - Standard laboratory procedures should be followed for perfusion fixation. Formaldehyde, paraformaldehyde or glutaraldehyde may all be used as fixatives. After 2 hours of tissue perfusion, the dissected tissue should be immersed in fresh 3.7% Buffered Formaldehyde for up to 24 hours.

Storage of Fixed Samples - Fixed samples may be stored for long periods. For long term storage, use 70% ethanol or sterile 1X PBS at 2-8 °C to avoid extended exposure to fixative. Archival material that has been stored in fixative for months or years will be more difficult to permeabilize and may not be useful for *in situ* detection of apoptosis due to DNA degradation.

Cryosection of Fixed Tissue - Immerse fixed tissue in 20% sucrose in water at room temperature until the sample sinks. Embed the cryoprotected sample in cutting matrix and freeze. Collect sections of 6-10 µm onto slides pretreated for electrostatic adherence of samples and dried as described in Preparation of Fresh Frozen Sections.
Note: *When collecting onto slides from buffer use either a low salt buffer or distilled water to ensure that samples adhere to slides.*

Storage of Sectioned Tissues and Rehydration - Sections of fixed frozen tissue may be stored at ≤ -70 °C, with desiccant, for up to one month. After storage, the slides should be equilibrated to room temperature and dried for 2 hours at room temperature or at 45 °C on a slide dryer. Rehydrate samples before labeling by immersing for 5 minutes each in 100%, 95%, then 70% ethanol and wash for 10 minutes in 1X PBS.

SAMPLE PREPARATION AND FIXATION *Continued*

Section Paraffin Embedded Tissues - Paraffin embedding is a routine procedure in many laboratories and is commonly performed by automated equipment. **The temperature of the molten paraffin must not exceed 65 °C**, otherwise additional DNA damage can occur leading to spurious positives and high background. Do not bake slides after sectioning.

Sections between 6-10 µm should be collected onto slides pretreated for electrostatic adherence. Prior to the labeling reaction the samples must be deparaffinized. Optimal labeling is achieved when the samples are processed within days of sectioning.

Deparaffinization of sections prepared from paraffin blocks is required prior to the labeling reaction.

1. Warm slides to 57 °C for 5 minutes.
2. Immerse sections in 2 changes of xylenes, 5 minutes each.
3. Immerse sections in 100%, 95% then 70% ethanol, 5 minutes each.
4. Wash 2 times in 1X PBS, 5 minutes each.
5. Proceed to Labeling Procedure.

Storage

It is preferable to store the uncut paraffin block at room temperature, as opposed to the sections.
Note: *The xylenes and ethanols used for deparaffinization can be reused several times (up to 100 slides may be processed in 200 mL) but they must not be used for rehydration of non-embedded samples or for dehydration after performing the labeling reaction.*

LABELING PROCEDURE

Details on the labeling procedure are provided in the table on the following page.

Labeling Samples on Slides - Wash slides in 1X PBS using small Coplin histology jars. Each jar holds up to 50 mL of buffer and up to 10 slides. For procedural steps involving 50 µL per sample, place slides on a flat surface and spot reagent from above using a pipette tip; do not touch the sample with the pipette tip. Small biopsy samples are easily covered with 50 µL. If 50 µL does not cover the sample, hydrophobic coverslips may be used after pipetting the 50 µL volume. Lower the hydrophobic coverslip from one edge and press down gently to expel any air bubbles. Remove hydrophobic coverslips by dipping the slide vertically in distilled water.

Labeling Samples in Chamber Slides - Remove chamber walls and gasket after fixation and process as described for slides. hydrophobic coverslips may be used for all steps involving 50 µL reaction volumes. If different labeling reactions are performed on samples on the same slide, leave the plastic walls in place until after the labeling reaction, then remove the plastic walls and rubber gasket and proceed.

LABELING PROCEDURE *Continued*

Labeling Samples on Glass Coverslips - Process the 12 mm glass coverslips with the cell-side facing up in the 24-well tissue culture plate. Wash by filling the wells with 1X PBS and removing with a pipette. Spot the 50 µL reaction volumes directly onto the coverslip. Alternatively, spot the 50 µL reaction buffers onto a clean glass slide, then remove 12 mm glass coverslip from the well and flip it over, cell-side down, on top of the reagent. Use fine tipped forceps and handle glass coverslips only at the very edges. For dehydration and clarification, dip the 12 mm glass cover slips individually in ethanol series and xylenes for 20 seconds.

Note: *Xylenes will melt plastics, therefore, do not add xylenes to tissue culture plates.*

Step	Instructions	Notes
1	Place samples in 1X PBS for 10 minutes at room temperature after rehydration in Ethanols. Carefully dry glass slide around sample.	DO NOT allow sample to DRY at any stage prior to completion of protocol.
2	Cover sample with 50 µL of Proteinase K Solution and incubate 15-30 minutes at room temperature, or cover sample with 50 µL of Cytonin and incubate for 15-30 minutes at room temperature or 2 hr at 2-8 ˚C. If necessary, use hydrophobic coverslips.	Cytonin is recommended for frozen sections and when protease treatment will destroy antigens of interest in double labeling experiments. Time of Proteinase K Solution treatment will vary between cell and tissue type. Start at 15 minutes and increase if no labeling occurs.
3	Wash 2 times in distilled water, 2 minutes each.	
4	Immerse slides in Quenching Solution for 5 minutes at room temperature.	Do not leave longer than 5 minutes since hydrogen peroxide can damage DNA.
5	Wash samples 2 times, 1 minute each at room temperature in distilled water to remove Quenching Solution.	
6	Immerse slides in 1X TdT Labeling Buffer for 5 minutes.	
7	Cover sample with 50 µL of Labeling Reaction Mix and incubate at 37 ˚C for 1 hour in a humidity chamber. If necessary, use hydrophobic coverslips.	Use humidity chamber during incubation time.
8	Immerse samples in 1X TdT Stop Buffer for 5 minutes at room temperature to stop labeling reaction.	
9	Wash samples 2 times in distilled water for 5 minutes each at room temperature.	
10	Cover sample with 50 µL of Strep-HRP Solution and incubate 10 minutes at room temperature. If necessary, use hydrophobic cover slips.	Use Blue Streptavidin-HRP Diluent to prepare Strep-HRP Solution.
11	Wash samples 3 times in distilled water for 5 minutes each at room temperature.	
12	Cover samples with 50 µL TACS Blue Label for 2-10 minutes.	
13	Wash samples in several changes of distilled water for 2 minutes each.	
14	Proceed to Counterstaining and Preparation for Viewing.	

LABELING PROCEDURE *Continued*

COUNTERSTAINING AND PREPARATION FOR VIEWING

Cells and tissues may be counterstained with Nuclear Fast Red. Glass coverslips can be held in fine tipped forceps and dipped individually into the stains and ethanols. Spot only 25 µL Permount™ Mounting Medium onto a clean glass slide and mount the coverslip, cell side down, onto the slide. If a plastic support was used for cell culture do not pass through xylenes.

1. Immerse samples for 30 seconds to 5 minutes in Nuclear Fast Red.
2. Wash slides sequentially by dipping ten times in distilled water.
3. Air dry or dehydrate by dipping ten times in 95% ethanol.
4. Clarify by dipping ten times, in two changes of xylene. Wipe off excess xylene from the back of the slide and lay slide flat.
5. Place one drop, about 25 µL, of Mounting Media from a 100 µL pipette onto sample.
6. Lower glass cover slip onto sample and apply gentle even pressure to expel air bubbles.
7. Leave slide flat overnight to allow Mounting Media to harden.
8. Blue Label and Nuclear Fast Red stained samples are stable for 1 year **in the dark.**

CONTROLS

If you wish to use a pre-prepared control you may use the Tissue Control Slides (R&D Systems, Catalog # 4800-30). These controls allow you to run through the procedure to become familiar with handling the samples, etc.

Each set of Tissue Control Slides is shipped with a product information sheet that provides information on the recommended permeabilization method, incubation times, and interpretation of data. It is critical to run controls using the provided TACS-Nuclease to assess and optimize cell/tissue permeabilization. The recommended experimental controls are listed below:

TACS-Nuclease-Treated Control Sample - Treat one sample with TACS-Nuclease to generate DNA breaks in every cell. Avoid repeated freeze-thaw cycles. The TACS-Nuclease-treated Control Sample will confirm that the permeabilization and labeling reaction has worked. The information can help optimize the conditions for the labeling procedure. The majority of cells should exhibit blue nuclear staining.

1. After Step 5 of the Labeling Procedure, wash 2 times in distilled water, 2 minutes each.
2. Prepare TACS-Nuclease Solution by diluting TACS-Nuclease 1:50 in the TACS-Nuclease Buffer.
3. Cover sample with 50 µL of TACS-Nuclease Solution.
4. Incubate at room temperature for 10-60 minutes in a humidity chamber. If necessary, use hydrophobic coverslips.
5. Wash 2 times in 1X PBS for 2 minutes each.
6. Continue from Step 6, Labeling Procedure.

Though the above method is recommended, to save time, TACS-Nuclease can be added directly to the labeling mix of the positive control sample. Add 1.0 µL of TACS-Nuclease to 50 µL of complete labeling mix and incubate for the regular labeling time. The signal intensity obtained, using this method, is usually lower than the recommended method.

Unlabeled Experimental Control Sample - The TdT Enzyme should be omitted from the Labeling Reaction Mix for one sample. This control will indicate the level of background labeling (TACS Blue Label) associated with non-specific binding of the Strep-HRP. This control should not have any blue staining.

Experimental Negative Control Sample - An appropriate experimental control should be included in each experiment and will depend upon the system under study. Typically, the experimental negative control will be an untreated sample, or normal cells/tissues. Many normal or untreated cells and tissues will have a small number of apoptotic cells so a few cells may be positive for blue staining.

CONTROLS *Continued*

Counterstaining Control Sample - Although uncommon, some cells and tissues may take up excessive amounts of the Nuclear Fast Red counterstain, obscuring the TACS Blue staining. It is recommended to process 1 or 2 samples up to and including the distilled water wash step after the quenching step (Step 5) of the Labeling Procedure. Process through counterstaining. Staining times of 2 seconds to 30 minutes have been noted. For most cells and tissues 1 minute is sufficient.

DATA INTERPRETATION

Apoptosis is often defined by morphological criteria. Morphological data obtained from standard microscopy and histochemistry should always be considered in conjunction with biochemical assays used to confirm apoptosis.

Nuclear Fast Red allows all cells in the specimen to be visualized. Cells that are condensed (pyknotic, mitotic or apoptotic) will exhibit increased Nuclear Fast Red uptake. Cells containing fragmented nuclear chromatin characteristic of apoptosis will exhibit a blue nuclear staining that may be very dark after labeling. This dark blue staining is typically associated with cell condensation. Blue staining in the cytoplasm as well as the nucleus of enlarged or swollen cells may occur in instances of necrosis. In tissue sections where cells have been torn open during sectioning or the edges of the specimen are ragged there may be non-specific blue staining that is not associated with nuclei.

The controls listed are important in data interpretation. These controls allow optimization of in situ detection of apoptosis without expending valuable test samples. Under optimal conditions the Unlabeled Experimental Control should show no blue staining, the TACS-Nuclease-treated Control Sample should show pale blue staining in almost all cells, and the Experimental Negative Control Sample should have less than 10% blue stained cells. The blue staining of TACS-Nuclease treated cells is paler and usually more diffuse than the staining of truly apoptotic cells. This is due to the difference in chromatin structure between nuclease treated normal cells and the fragmented chromatin of apoptotic cells. The Counterstain Control Sample should show pale red/pink staining of all cells with some variability in intensity between cell types and darker staining of any condensed cells within that sample. Eosinic cells will take up more Nuclear Fast Red. Refer to the Troubleshooting Guide for information if the controls do not provide the expected result.

TROUBLESHOOTING

Rule out major problems by checking the labeling in the control samples first.

Problem	Cause	Action
No labeling in experimental sample.	No apoptosis (or necrosis) occurring in sample.	If all controls gave the expected results and were processed at the same time as the experimental sample there may be no DNA fragmentation in cells within the sample.
Blue staining of cells when the TdT Enzyme is omitted from the Labeling Reaction Mix.	Endogenous peroxidase activity inadequately quenched.	Use fresh aliquots of 30% hydrogen peroxide.
	Excessive peroxidase activity in sample (rare).	Increase concentration of hydrogen peroxide in Quenching Solution to 5%.
	Non-specific binding of Strep-HRP.	Increase number of washes after binding.
		Prepare Strep-HRP Solution in 1X PBS, 1% BSA.
		Decrease concentration of Strep-HRP by diluting stock solution up to 1:1000.
	Sample dried out during the labeling procedure.	Use hydrophobic coverslips (or lids for plates or chamber slides) and incubate in humidity chamber.
No staining in TACS-Nuclease-Treated Control Sample.	Poor permeabilization and/or excessive fixation with cross-linking fixative (common with archival tissue) preventing enzyme access.	Increase incubation time with Proteinase K Solution (up to 60 minutes) or Cytonin (up to overnight at 2-8 ˚C).
	No DNA left in sample due to hydrolysis (poor storage of samples or sections).	Read Sample Preparation and Storage of Samples prior to labeling.
	Excessive (removed all DNA) or inadequate TACS-Nuclease treatment.	Optimize time for TACS-Nuclease treatment (5 minutes up to 2 hours).
	TdT Enzyme is inactive. The enzyme is the most labile component in the kit.	TdT Enzyme must be stored at ≤-20 ˚C in a manual defrost freezer. Do not bring enzyme up to ice temperature. Place in -20 ˚C block or remove aliquot from tube directly in freezer.
	Mixed xylenes were used during clarification and TACS Blue Label was solubilized.	Use only o- or p-xylene that do not contain contaminating benzenes.
Labeling of majority of cells in the negative experimental control (i.e. normal tissue or untreated cells) when there is no labeling if the enzyme is omitted and satisfactory labeling of the Nuclease-treated control.	High level of apoptosis (or necrosis) in negative control.	Select a more appropriate negative control or inhibit apoptosis in cell culture (i.e. with protein synthesis inhibitors). Check morphology of cells prior to assay for evidence of excessive apoptosis.
	Prolonged incubation with Blue Label leads to a precipitate over entire sample.	Reduce time in TACS Blue Label and follow color development under the microscope.
	Excessive Proteinase K treatment.	Reduce incubation time in Proteinase K Solution to 5-15 minutes, or dilute Proteinase K Solution 1:200 in water and incubate sample for 15 minutes.
Experimental sample shows extensive cytoplasmic staining.	High rate of cell death, late apoptosis or necrosis.	Necrotic samples will exhibit cytoplasmic staining. Apoptosis in cell culture will progress to necrosis. Reduce time of treatment in cell culture.
Intense Red Counterstain.	Overstaining.	Reduce time in Nuclear Fast Red.

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